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14. ABSTRACT

Membrane-type serine protease 1 (MT-SP1) is a cancer-associated serine protease implicated in the tumorogenesis and metastasis of breast cancer. Inhibition of MT-SP1 activity has been shown to decrease metastatic potential. We have developed a number of potent and specific single-chain (scFv) antibody inhibitors to MT-SP1, and have begun to characterize their mechanism of inhibition. Through kinetic characterization and site-directed mutagenesis experiments, it has been determined that three potent inhibitors have separate and novel mechanisms of inhibition which do not mimic either biologically or pharmaceutically relevant protease inhibitors. These novel modes of binding and inhibition are the basis for their specificity, and suggest these inhibitors will have less cross-reactivity and toxicity problems when used in vivo to further dissect the role of MT-SP1 in breast cancer.

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Introduction:

My research has focused on the mechanism of inhibition of a set of single-chain inhibitors of Membrane-Type Serine Protease 1 (MT-SP1). MT-SP1 is a type II transmembrane serine protease (TTSP) expressed on the surface of epithelial cells. It was discovered and cloned in a search for serine proteases expressed in PC-3 cells, a prostate cancer cell line (Takeuchi et al. 1999), and was independently determined to be a highly expressed protease in breast cancer tissue (Lin et al. 1999). Immunoblotting, immunohistochemical analysis, and expression level analysis have found MT-SP1 to be differentially overexpressed in breast, prostate, and ovarian cancers. MT-SP1 has been shown to play a role in ovarian (Suzuki et al. 2004) and prostate (Galkin et al. 2004) tumor invasion using experimental methods including inhibition of MT-SP1 by small molecules and anti-sense. In breast cancer, high levels of MT-SP1 expression has been correlated with the expression of hepatocyte growth factor (HGF) and the Met/HGF receptor, and overexpression of these components is prognostic of patient mortality (Kang et al 2003). Most recently, modest orthotopic overexpression of MT-SP1 in mouse epidermal tissue led to spontaneous squamous cell carcinomas (List et al. 2005), further cementing MT-SP1's role in cancer, and suggesting the enzyme is causally involved in malignant transformation.

In order to tease apart the role of MT-SP1 in tumor progression, the Craik Lab has used phage display to develop a series of potent and specific single-chain antibody inhibitors (scFv) of the catalytic domain of MT-SP1 (Sun et al. 2003). With K_i's ranging from 10pM to 10nM, these inhibitors are extremely potent *in vitro*, and showed no appreciable inhibition of a panel of closely related serine proteases including factor Xa, thrombin, kallikrein, tPA, and uPA. The potential benefits of these inhibitors are two-fold: they can be used to probe complex biology of MT-SP1, both its role in normal and cancer biology, and they can be used to validate MT-SP1 as both an imaging and therapeutic target. From a more biophysical standpoint, these inhibitors are unique in that they are the only reported antibody inhibitors of serine proteases, a large class of homologous enzymes in which the development of specific inhibitors has been a monumental challenge. Most protease inhibitors take advantage of either the catalytic machinery or topological fold of the protease. These scFv inhibitors bind and recognize a specific three-dimensional epitope near the active site of the enzyme, which allows for specificity among proteases, and allows for a fundamentally different mechanism of inhibition from other biologically active protease inhibitors. A thorough understanding of the mechanism of inhibition of these inhibitors will help us validate their putative mode of action *in vivo*, and will suggest new strategies for inhibition of MT-SP1 and other serine proteases.

Results:

My proposal's specific aims were to kinetically and structurally characterize the interactions between three potent scFv inhibitors of MT-SP1, named EB-9, SA-12, and TD-10. The results of the kinetic experiments to date are shown in Table I. From the data in Table I, it is clear that the three inhibitors are reversible, competitive, tight-binding inhibitors of MT-SP1. Furthermore, the inhibitors exhibit the same mode of inhibition with similar K_i's when a macromolecular substrate, single-chain uPA, is used instead of the traditional small molecule substrates used to study proteases. This is significant in that it suggests that these inhibitors will inhibit MT-SP1 in a similar manner *in vivo* when the protease comes in contact with its natural substrates.

Table I: Kinetic Parameters of scFv Inhibitors

	k_{on}* ($10^6 M^{-1}s^{-1}$)	k_{off}* ($10^{-3}s^{-1}$)	K_d* (nM)	Mode of Inhibition	K_i (pM)	Macro molecular MOI	Macro molecular K_i (pM)
EB-9	2.1	0.38	0.16	Competitive	10.5	Competitive	??? (low)
SA-12	1.5	1.9	1.3	Competitive	10,000	Competitive	2000
TD-10	11.5	5.8	0.51	Competitive	131	Competitive	160

*Values Determined by SPR. Sun *et al.* 2003

Table I: **Kinetic Parameters of scFv inhibitors.** K_d's, k_{on}'s and k_{off}'s were measured by SPR. Mode of inhibition and K_i's were measured by solution kinetics, and macromolecular mode of inhibitions and K_i's were measured by a linked enzyme assay in which MT-SP1 activates pro-uPA, whose rate of activation is measured.

The kinetic parameters for the inhibitors are consistent with those of most macromolecular protease inhibitors and antibody-antigen interactions. The on rate of TD-10 is an order of magnitude faster than the other inhibitors, which suggests that electrostatic steering plays an important role in the interaction. The range of kinetic parameters does suggest that multiple mechanisms of inhibition for the panel of inhibitors. The detailed kinetic mechanism will be studied using stopped-flow kinetics shortly.

My proposal aimed to crystallize the MT-SP1-inhibitor complex of all three inhibitors. To date, despite setting up more than 1,500 crystallization conditions, I have not been able to get crystals that diffract to more than 8 angstroms. In lieu of structural data, I have begun to use site-directed mutagenesis to determine the binding epitopes of the inhibitors. To do so, I mutate residues on the surface of the protease to alanine, and measure the K_i's of the inhibitors to the mutant proteases. The mutations characterized so far have not had an appreciable affect on proteolytic activity. To date, I have made and characterized 13 different MT-SP1 mutants and found unique binding epitopes for each inhibitor.

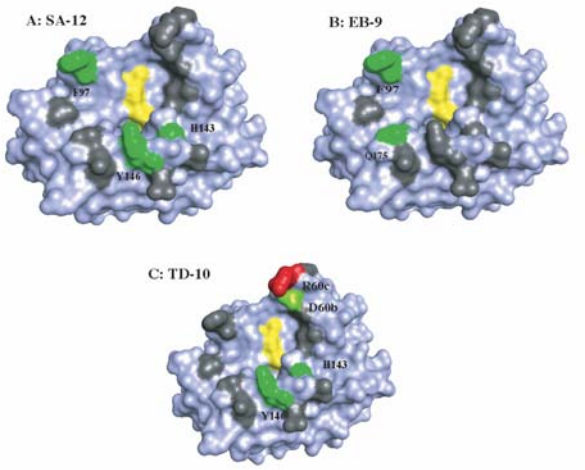

Figure 1**: Binding footprints of antibody inhibitors mapped to the catalytic domain of MT-SP1.** The catalytic triad of MT-SP1 is colored yellow. Residues colored green deleteriously affected protease inhibition by the inhibitors when mutated to alanine. Residues colored red improved inhibition when mutated to alanine. Mutations of gray residues had no affect on inhibition when mutated to alanine.

For reference, I have also tested these mutants against bovine pancreatic trypsin inhibitor (BPTI), a known serine protease with a well-studied mechanism of inhibition. The results are summarized in Table II, and mapped to the crystal structure of the protease in Figure 1. Each inhibitor has a unique binding epitope on the surface of MT-SP1, which is different than all known protease inhibitors. Two of the inhibitors, SA-12 and TD-10, bind to the surface loops flanking the protease active site, and prevent substrate binding. This helps to explain the selectivity of the inhibitors: while the catalytic machinery and the fold of all trypsin-like serine proteases are identical, the surface loops have a high degree of sequential diversity, and these inhibitors recognize the sequentially diverse portions of MT-SP1, rather than the catalytic machinery. In this regard, MT-SP1 is an ideal candidate for inhibition by scFv's because it has large surface loops, larger than those found in most serine proteases.

Table II: Point Mutant / Inhibitor IC_{50} Values

	BPTI		EB-9		TD-10		SA-12	
	IC_{50} (nM)	Fold Difference	IC_{50} (pM)	Fold Difference	IC_{50} (pM)	Fold Difference	IC_{50} (nM)	Fold Difference
MT-SP1	6.8		340		867		22	
D60bA	250	37	1250	3.7	3540	4.1	13	0.6
R60cA	2.5	0.36	190	0.56	56	0.065	15	0.7
R60fA	10.5	1.5	176	0.52	1090	1.3	37	1.7
Y60gA	3.2	0.47	375	1.1	1180	1.4	55	2.5

	BPTI		EB-9		TD-10		SA-12	
	IC_{50} (pM)	Fold Difference	IC_{50} (pM)	Fold Difference	IC_{50} (pM)	Fold Difference	IC_{50} (nM)	Fold Difference
MT-SP1	449		126		577		10	
I41A	127	0.28	138	1.1	1285	2.2	20	2
Q145A	426	1	280	2.2	448	0.8	16	1.6
Y146A	505	1.1	482	3.8	6397	11.1	8500	850
D217A	5000	11.1	170	1.3	1900	3.3	28.9	2.9

	BPTI		EB-9		TD-10		SA-12	
	IC_{50} (pM)	Fold Difference	IC_{50} (pM)	Fold Difference	IC_{50} (pM)	Fold Difference	IC_{50} (nM)	Fold Difference
MT-SP1	370		295		537		10	
H143A	174	0.47	282	0.96	60000	111	206	20.6
Q175A	350	1	7500	25.4	2000	3.7	15	1.5
Q221aA	367	1	234	0.8	380	0.7	10	1
F97A	1600	4.3	>1uM	$>10^7$	1100	2	200	20
T150A	412	1.1	286	1	681	1.3	15	1.5

TABLE II: **IC_{50} Values of scFv Inhibitors and MT-SP1 Point Mutants**. The point mutants that increased inhibitor IC_{50} are highlighted in green, those mutations that decreased the IC_{50} are in red. The IC_{50} value had to differ by more than 4-fold to be considered real.

The mutational analysis of EB-9 inhibition of MT-SP1 is also striking. The mutation of phenylalanine 97 to alanine (F97A) increased the K_i of EB-9 by more than 10^5-fold. This suggests that EB-9 derives much of its binding energy from an interaction with F97 on the protease. Located near the extended substrate-binding pocket of MT-SP1, F97 is part of a patch containing a number of aromatic residues, creating a hydrophobic patch on the surface of the protease likely involved in macromolecular substrate binding. This therefore suggests EB-9 has bound to a secondary substrate-binding site, apart from the catalytic residues and S1 pocket. This mechanism, binding to secondary sites on the protease surface, has been seen in some inhibitors, such as the leech-derived thrombin inhibitor, but the exosite is always used as a secondary binding site, and the inhibitor binds in the active site as well. As the binding footprint and kinetics of EB-9 are completed, it will be interesting to see if the inhibitor binds in the active site as well, and uses a similar mechanism as those inhibitors under evolutionary pressure to be specific for a single protease.

Key Research and Training Accomplishments:

- Determined K_i's and mode of inhibition for the panel of scFv inhibitors of the breast-cancer associated serine protease MT-SP1 (Table I).

- Have begun to map out the binding site of the inhibitors on the serine protease surface through site-directed mutagenesis. This has revealed novel binding sites for protease inhibition, and suggests novel mechanisms of inhibition that could help specifically inhibit specific members of the trypsin-fold serine protease family.

- Attended the UCSF Breast Oncology Program annual conference. This two-day retreat had an intense focus on the epidemiological basis of breast cancer, methods of early detection and the effort underway to translate basic research to the clinic.

- Presented peer-reviewed posters summarizing this work at the UCSF Breast Oncology Program and UCSF Biophysics/CCB Retreat.

Reportable Outcomes:

All results summarized above are reportable, and will be packaged into a paper when the experiments are completed.

References:

Galkin AV, Mullen L, Fox WD, Brown J, Duncan D, Moreno O, Madison EL. CVS-3983, a selective matriptase inhibitor, suppresses the growth of androgen independent prostate tumor xenografts. *The Prostate* 2004, **61,** 228-35.

Kang JY, Dolled-Filhart M, Ocal IT, Singh B, Lin CY, Dickson RB, Lim DL, Camp RL. Tissue microarray analysis of hepatocyte growth factor/met pathway components reveals a role for met, matriptase, and HGF-HAI1 in the progression of node-negative breast cancer. *Cancer Research* 2003, **63**, 1101-5.

Lin CY, Anders J, Johnson M, Dickson RB. Purification and characterization of a complex containing matriptase and a Kunitz-type serine protease inhibitor from human milk. *Journal of Biological Chemistry* 1999, **274**, 18237-42.

List K, Szabo R, Molinolo A, Sriuranpong V, Redeye V, Murdock T, Burke B, Nielsen BS, Gutkind JS, Bugge TH. Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. *Genes Dev* 2005, **16**, 1934-50.

Sun J, Pons J, Craik CS. Potent and selective inhibition of membrane-type serine protease 1 by human single-chain antibodies. *Biochemistry* 2003, **42**, 892-900.

Suzuki M, Kobayashi H, Kanayama N, Saga Y, Suzuki M, Lin CY, Dickson RB, Terao T. Inhibition of tumor invasion by genomic down-regulation of matriptase through suppression of activation of receptor-pound pro-urokinase. *Journal of Biological Chemistry* 2004, **279**, 14899-908.

Takeuchi T, Shuman MA, Craik CS. Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc Natl Acad Sci U S A* 1999, **96**, 11054-61.